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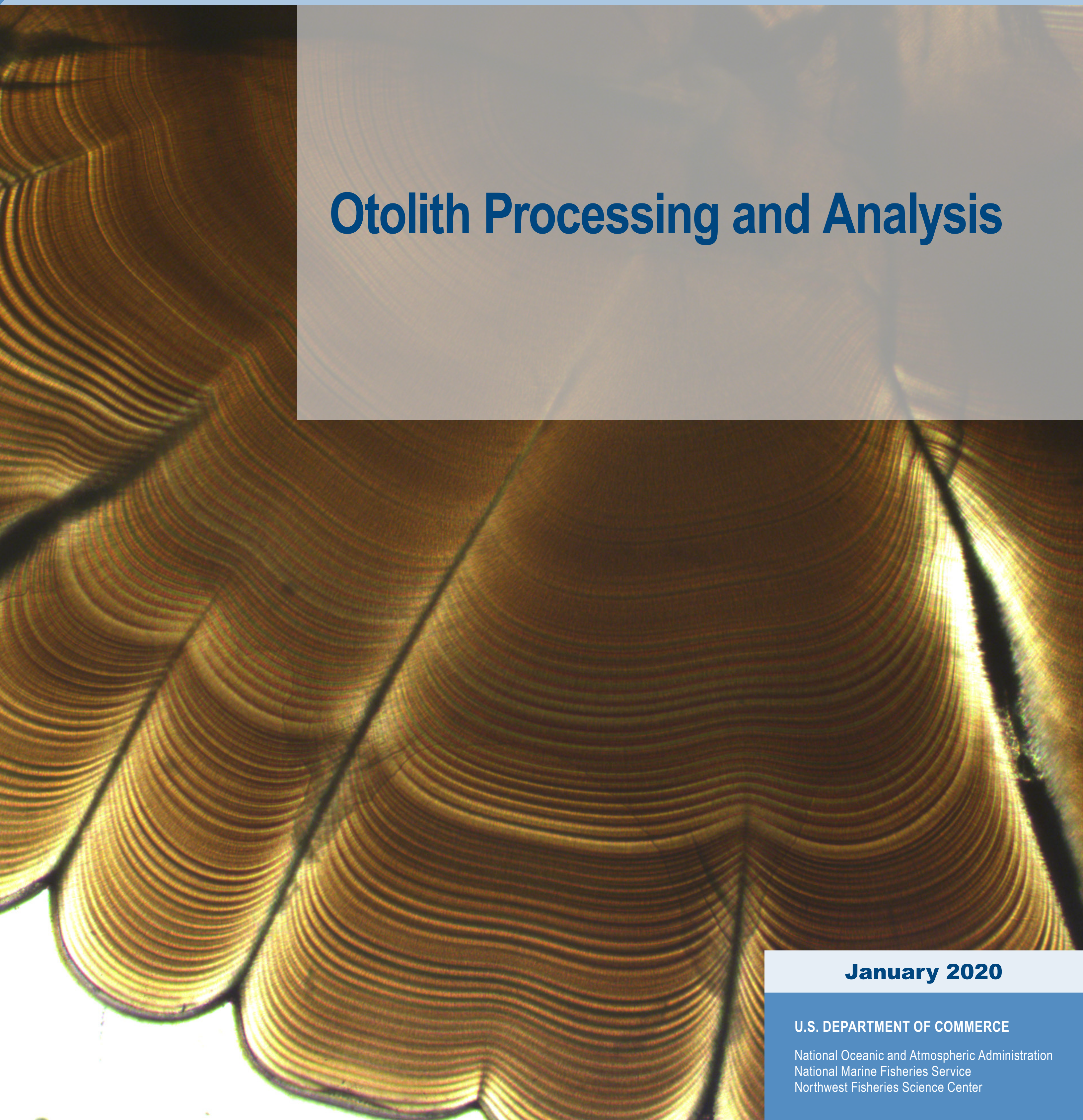

Otolith Processing and Analysis

January 2020

U.S. DEPARTMENT OF COMMERCE

National Oceanic and Atmospheric Administration
National Marine Fisheries Service
Northwest Fisheries Science Center

NOAA Processed Report Series NMFS-NWFSC-PR

Cover photograph courtesy of K. Veggerby, NMFS/NWFSC.
Detail of a juvenile fish otolith.

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Otolith Processing Protocol

How to prepare juvenile salmonid sagittal otoliths for microstructure analysis.

Otolith lab supplies

Dissecting materials and otolith storage supplies:

- Dissecting microscope with an eye micrometer and light source
- Scalpel
- Latex or nitrile gloves
- Probe
- Scissors
- Forceps
- Otolith storage containers: microcentrifuge vials (size: 1.0 or 1.5 mL, color: light colors, preferably clear) or small glass vials
- Storage identification labels

Embedding materials:

- Large q-tips
- Epoxy resin
- Resin catalyst
- Crystalbond glue
- Embedding trays
- Silicon spray
- Probe
- Alcohol/acetone for cleaning
- Oven (for baking/setting the epoxy)
- Pipettes

Otolith mounting materials:

- Hot plate
- Microscope slides
- Dissecting microscope and light source
- Glass etching tool
- Containers to store microscope slides
- Probe
- Forceps
- Syringe (small with a very fine tip; they can be useful when working with small otoliths)
- Buehler Isomet Precision Saw (Figure 1)
- Buehler cutting fluid (lubricant for the saw)
- Buehler saw cleaning solution

Figure 1. Buehler Isomet Precision Saw used to cut epoxy resin while processing otolith samples.

Grinding materials:

- Grinding machines (Figures 2 and 3)
- Plates (Figure 4)
- Grinding jig (Figure 4)
- Light microscope (Figures 2 and 3)
- Grinding pads
    - Buehler pads: “MicroCloth PSA” and “TexMet PSA”
- Paper towels (washcloths also work)
- Wash bottles for slurries
- Beakers with water to rinse slides
- Cuff length nitrile gloves
- Grit (used to make grinding slurries)
    - Purchased from Buehler
    - Grit sizes (in microns): 30, 15, 5, and 1.0 alumina micropolish
    - We use different grit sizes because they each grind otoliths at different speeds. 30-micron is the largest grit (the size of each particle is large) and quickly grinds the otoliths. The 15-micron grit is smaller than 30-micron, and smaller still is the 5-micron grit. Both 15 and 5 grits are great to use because they slowly grind away otolith material, unlike 30 which can quickly ruin an otolith if you’re not attentive while grinding. The 1.0-micron grit is used only to polish the otolith surface. It does not grind away otolith layers; instead, it buffs the otolith surface and improves increment clarity.

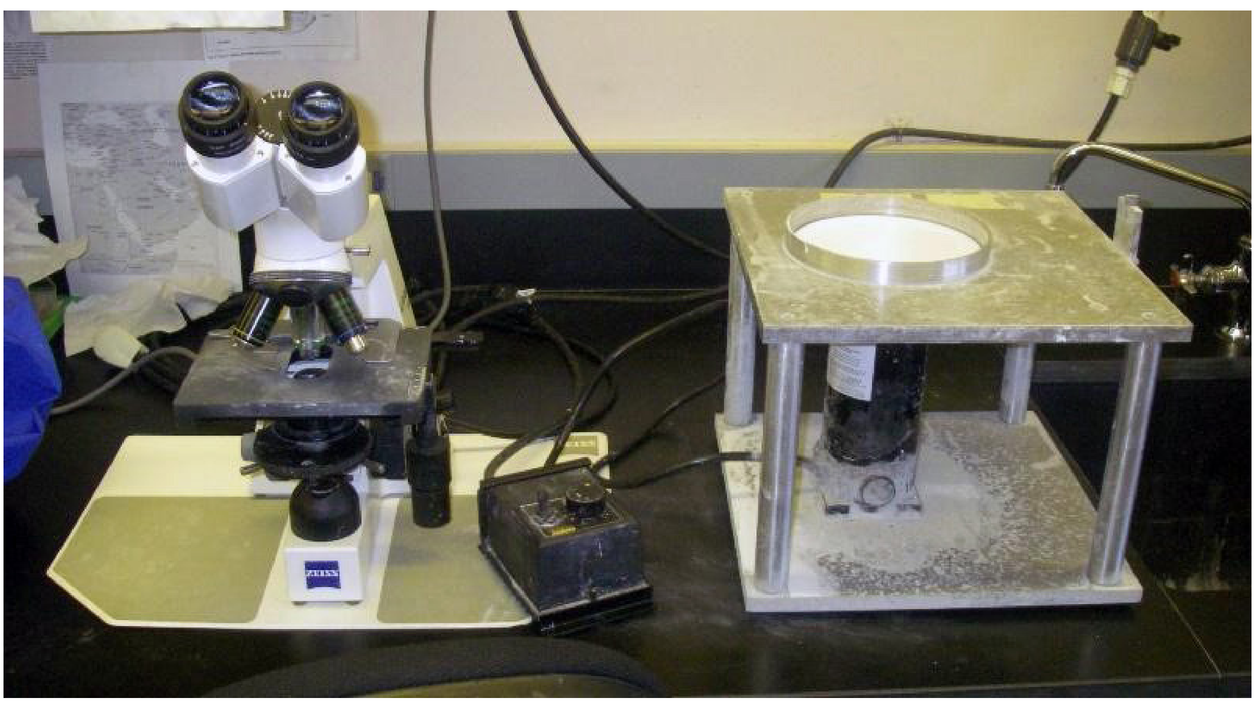

Figure 2. Laboratory equipment used to process otoliths: A light microscope (left) and an otolith grinding machine (right).

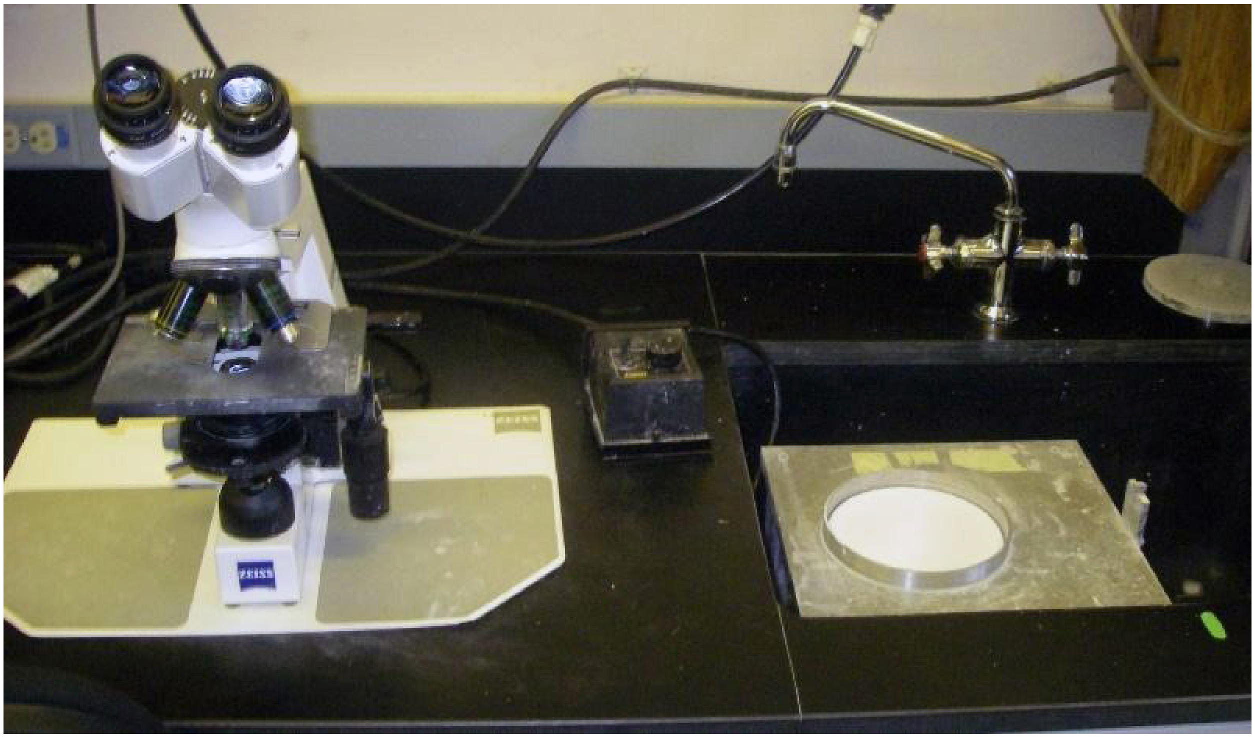

Figure 3. Laboratory equipment: The otolith grinder is placed inside the sink while processing otoliths in order to contain the mess created by the polishing slurry.

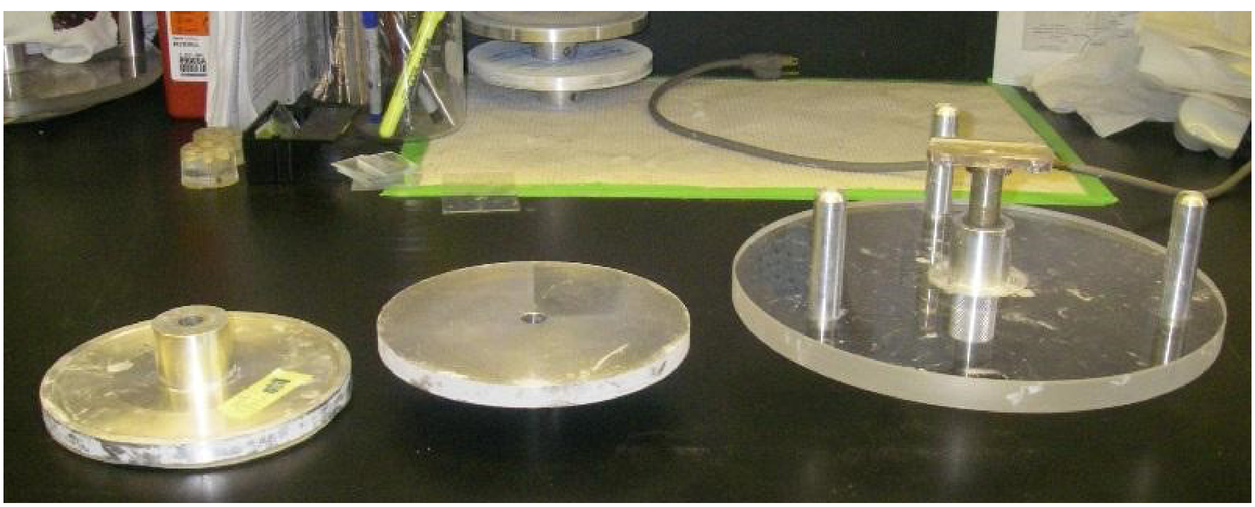

Figure 4. Two grinding plates (left) and a grinding jig (right).

Otolith analysis materials:

- Light microscope with camera attached (Figure 5).
- Computer with Image Pro software installed (Figure 5).
- Ruled stage micrometer (purchased from Ward's Natural Science)
- MS Excel or another spreadsheet application
- Image analysis software (Image J or Image Pro)

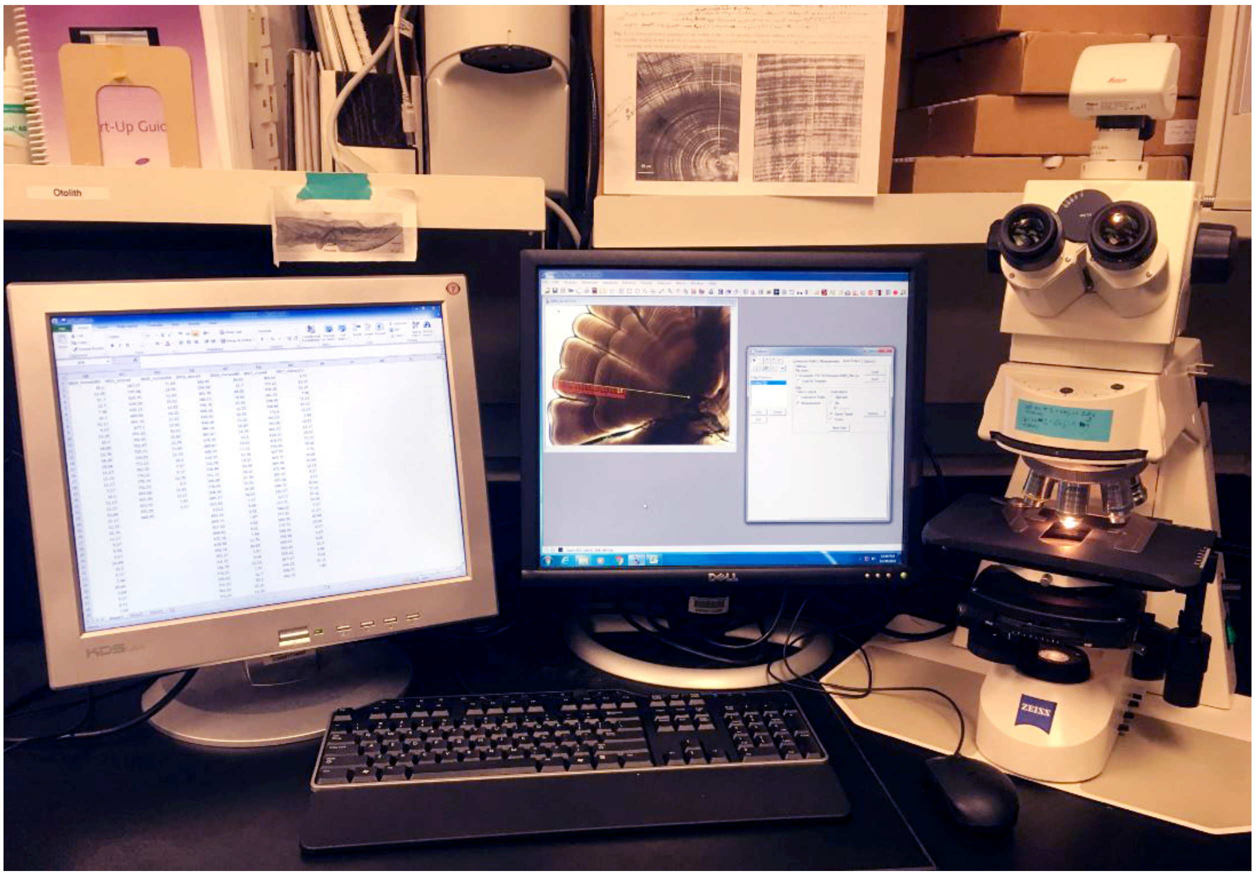

Figure 5. Image analysis workstation.

Otolith storage

If possible, try to prepare your otolith storage vials before you begin extracting them from the fish samples. To minimize storage space, I prefer to store otolith samples in microcentrifuge vials (1.0 mL or 1.5 mL).

1. Create otolith identification labels to be stored with each otolith within the storage vials. Each identification label should include the fish sample ID and sampling information (sample date, sample site). If the left and right otoliths are stored separately, make sure to include this on the label. It's helpful to use small strips of paper as labels (either printed from a computer or hand-written), as they can be easily read through the vial.
2. If you use microcentrifuge vials for storage, use a marker to label each vial with the fish sample ID and sample year.

Otolith removal

There are many different ways to cut a fish head and expose the brain cavity to extract otoliths; this protocol presents one such method.

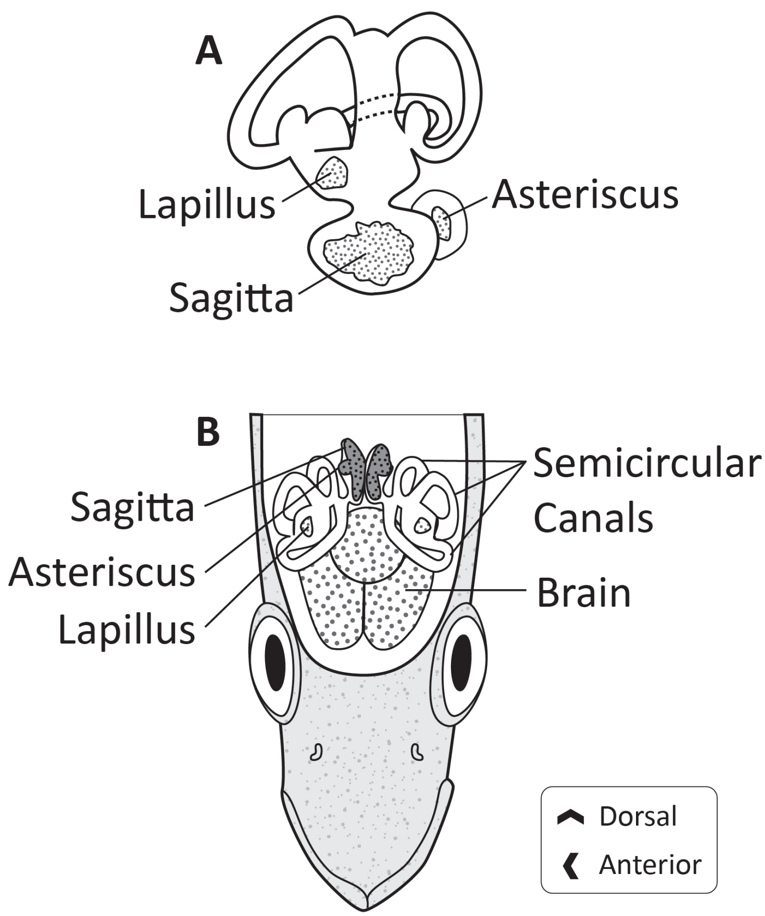

Figure 6. Anatomy of vestibular apparatus (inner ear structure). A) Otoliths within the labyrinth systems of a representative teleost fish (modified from Secor et al. 1991). B) Dorsal view of the vestibular apparatus as it sits in a typical teleost. Top of head is cut away.

Otolith removal protocol

1. If the fish head is frozen, allow time to thaw. It is easy to break or damage an otolith if you try to remove it from a frozen fish head. Speed up thawing by holding the fish head in your hand or by placing your finger on top of the brain cavity.
2. For removing otoliths from juvenile fish, I prefer using a scalpel to slice off the top of the head. Begin slicing slightly behind (posterior) of the eyes, where the head begins to flatten out—it appears a little less curved.
3. Pull the sliced layer back toward the caudal fin. The brain cavity should now be exposed.

Note: If the fish is very small, it can be helpful to use a dissecting microscope and light source for the following steps.

4. Use forceps to remove the brain matter (white, mushy material).
5. The otoliths (sagittae) are tucked in separate cavities on the left and right sides of the brain (Figure 6). In these cavities the otoliths are packaged within a membranous, fluid-filled pocket. The otolith cavities are located at the bottom, posterior portion of the brain area. Use fine forceps to probe and extract the otoliths.

Note: Be careful as you probe and extract the otoliths. They are quite fragile and can break easily.

6. The membranous tissue surrounding each otolith may stick to the otolith as it is removed from the fish head. Gently remove the tissue from the otolith by softly rubbing your finger over the otolith. It is helpful to remove the membranous tissue immediately, because as the tissue dries it hardens and later may cause the otolith to chip while grinding.
7. Place the otoliths into labeled vials. Sometimes it is easier to store the left and right otoliths separately, in two separate vials—do whatever works best for your setup.
8. In terms of deciding which otolith to grind and use for microstructure analysis, we typically use the left otolith for microstructure and the right otolith for microchemistry analysis.

Note: The common calcium carbonate crystal structure within an otolith is called aragonite. At times (for unknown reasons), vaterite, another structural form of calcium carbonate, can develop as well. Areas of vaterite are easy to identify: vaterite appears opaque and, often, increments aren't visible within such regions. Because increments aren't present (or, if present, are severely altered), it's important not to measure otoliths within areas that contain vaterite.

Identifying left and right otoliths: A generalized description

An otolith has 2 sides, a sulcus and a non-sulcus side (Figure 7).

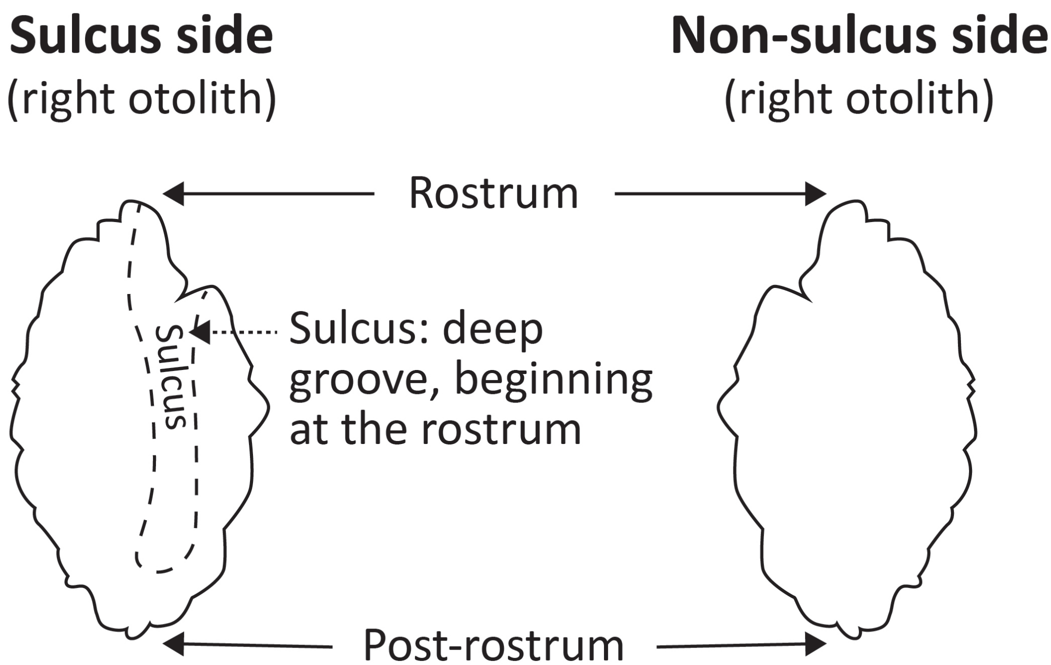

Figure 7. Sulcus and non-sulcus side views of a right sagittal otolith. Otolith features (sulcus, rostrum, post-rostrum) are labeled.

1. The sulcus is a deep groove located on the surface of the otolith. The sulcus is only on one side of the otolith, referred to as the sulcus side. The shape of the sulcus can vary among individuals and species. Regardless of its shape, the sulcus is often easy to identify because it is a distinct mark that looks as though a vertical strip has been carved out of the otolith surface.
2. To identify the left and right otoliths, look at the non-sulcus side of the otolith with the rostrum pointed away from you. Is the longest otolith edge located on the left or right side? If the longest edge is on the left side of the otolith, then it is a left otolith; alternatively, if the longest edge is on the right side of the otolith, then it is a right otolith. For example, look at the otolith on the left side in Figure 8. Its longest edge is located on the left side of the otolith, indicating that it is a left otolith.
3. Please note that this is a generalized description of juvenile otoliths. As previously mentioned, otolith morphology is variable, even among individuals within the same population. Otoliths from very young fish can be particularly challenging to identify because sometimes they are circular in shape, lacking a distinct rostrum. In such cases, identifying the left and right is easier if you know which side of the fish head each otolith was extracted from.

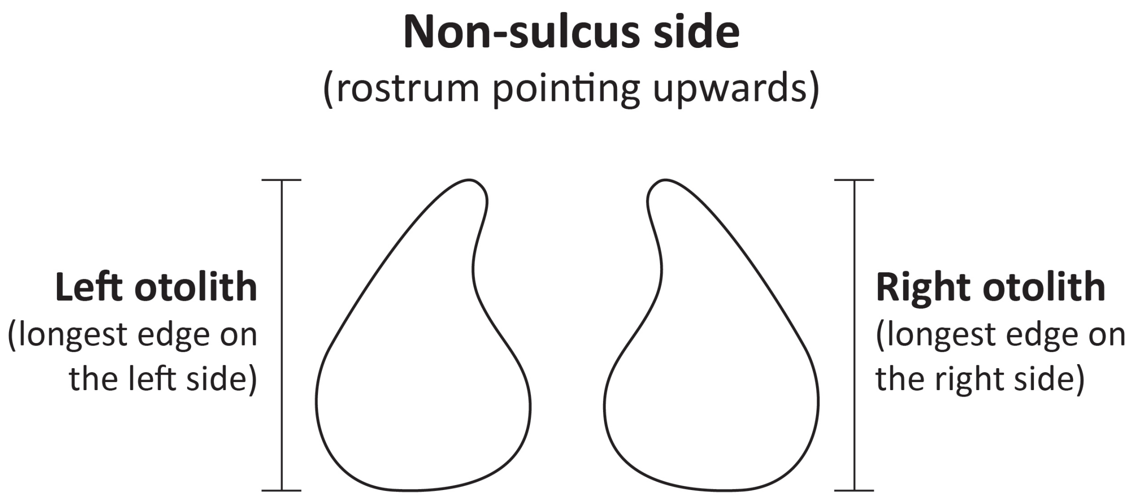

Figure 8. Non-sulcus side views of left and right sagittal otoliths. When viewing the non-sulcus side, the longest edges can be used to identify the right and left otoliths.

Otolith mounting

There are two ways to mount otoliths onto slides:

1. Embed the otolith within an epoxy resin block, then glue the resin block onto a microscope slide.
2. Crystalbond (CB) the otolith directly onto the microscope slide.

Both methods work fine, though the CB method is faster. It is debatable whether or not the resin block helps keep the otolith more flat while grinding (sometimes I think the resin block helps while grinding the first side of the otolith, other times it doesn't seem to make a difference).

Embedding with epoxy resin

Note: If you choose not to embed, skip this section and proceed to <u>Slide Preparation Step B</u>.

1. Use a marker to label each well within a small molding tray with the appropriate otolith sample IDs.
2. Use a swab to apply a thin coat of silicon on all sides of each well. The silicon helps the resin block release from the well when dry (essentially we're greasing the wells, similar to greasing a cake pan).
3. Place each otolith horizontally within a well (sulcus side down).
4. (*Caution*: Epoxy resin and its catalyst contain nasty chemicals. Make sure to do the following steps under a fume hood and do not inhale the vapors. Some people prefer to use a mask during this process.) Mix the resin and catalyst: For 20 otoliths use ~½ oz of resin and add ~3–4 drops of catalyst. Gently stir the catalyst and resin using a glass pipette (try to limit the number of air bubbles that form in the resin/catalyst mixture by not lifting the pipette up and down as your stir the mixture).

5. With a pipette, gently drizzle resin into the wells over the otoliths or in a corner of the wells (be careful not to move the otolith).
6. Fill each well about half full (be certain that the otoliths are completely covered).
7. To eliminate air bubbles and ensure that the otolith is properly situated, gently tap each otolith to the bottom of the well with a dissection probe. Try to press down vertically on the otolith, so you don't angle or alter its orientation.
8. Place the molding trays in a drying oven for 1 hour at 62°C. Store the drying oven in the hood to avoid exposure to vapors.

Slide preparation

A. For embedded otoliths (epoxy resin method)

1. Use a glass etching tool to label microscope slides with otolith ID and sample year; see Figure 9.
2. Turn on the hot plate (low setting, #3–4). Begin melting CB by placing several small pieces onto an unlabeled slide on the hot plate.
3. With a sharpie, write the sample number directly onto each resin block. This way, when you remove the resin blocks they won't get mixed up. Also, it will help when you orient the resin blocks onto the slides (especially with small samples). Remove all resin blocks from the molding trays.
4. Place a couple of slides across the front edge of the hot plate.
5. Use a dissection probe to apply CB to the heated, prelabeled slides. Do this by dipping the probe into the melted CB and allow the CB to harden on the probe (it will be helpful in step #6 if you shape the CB into a point as it hardens). After the CB has solidified on the tip of the probe, draw a rectangle on the labeled slide (do this while the slide is on the hot plate)—the CB will melt onto the slide as you draw the rectangle; see Figure 9. After you've made a rectangle of melted CB, remove your

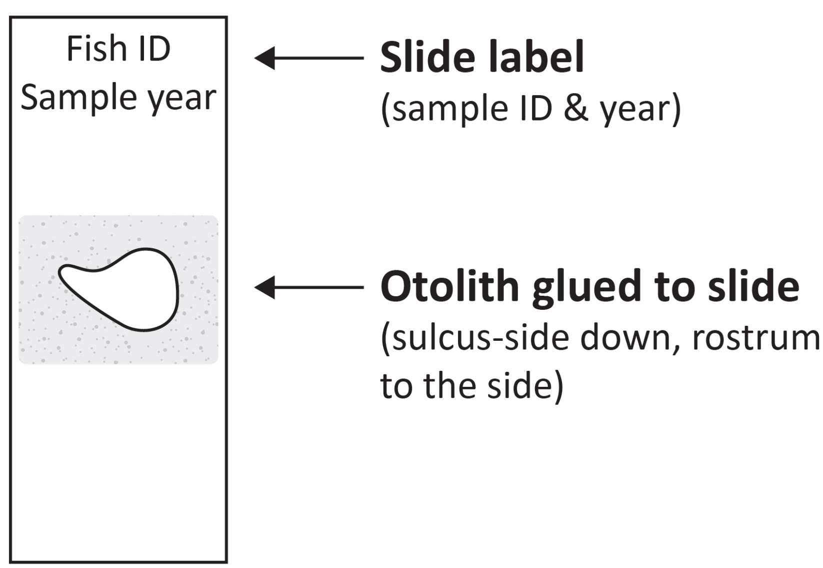

Figure 9. Microscope slide with sample label and otolith adhered to the surface.

slide from the hot plate and adhere the resin block to the CB. Lay the flat side of the resin block onto the CB (the resin block should be oriented the same way as it was in the molding tray, with the otolith sulcus side down and the rostrum pointing to the side). Apply a little pressure to ensure the resin block is firmly glued to the slide.

6. Before you begin grinding, use the lab saw to remove excess resin atop the otolith. To do this, place the slide in the saw jig, and laterally move the jig to a position where you will saw away as much resin as possible without the blade touching the otolith. Set the saw speed at 100–125. Avoid using a higher speed because it will cause the saw to cut through the resin block at an angle (thus, the top of the resin block will not be a flat surface on which to grind).

Note: Check the lubricant/water solution each time you use the saw. At minimum, there should be enough solution so that the saw blade is touching the surface of the solution. Remember to occasionally sharpen the saw blade with a sharpening stone. A sharp blade will quicken the sawing process.

B. For otoliths that are not embedded (Crystalbond only)

1. Use a glass etching tool to label microscope slides with otolith ID and sample year; see Figure 9.
2. Turn on the hot plate (low setting, #3–4). Begin melting CB by placing several pieces of CB onto an unlabeled slide on the hot plate.
3. Place a couple of slides across the front edge of the hot plate.
4. With the appropriately labeled slide in front of you on the hot plate, remove the otolith that you plan to mount and carefully place it on the lab bench. Using a dissection probe, apply CB to the heated, labeled slides. Do this by dipping the probe into the melted CB and allowing it to harden on the probe (as it hardens you can shape it into a point—this will be helpful in the next step). Make a small drop of melted CB on the slide.
5. Once the CB is melted on the labeled slide, remove the slide from the hot plate and place it on the lab bench. Using fine forceps or the tip of a small syringe, carefully (so as not to break the otolith) place the otolith into the drop of melted CB, sulcus side down with the rostrum pointing to the side. Gently push the otolith down through the melted CB so the sulcus is touching the slide surface. (For this last step, I find it helpful to view the otolith through a dissecting scope.) If the CB hardens before final positioning of the otolith, simply return the slide to the hot plate to remelt the CB—repeat as necessary.
6. Make sure the otolith is covered by CB—even a thin layer of CB on top of the otolith will suffice. If not completely covered with CB, the otolith can be ripped off the slide during the grinding process.

Grinding and polishing

A. How to prepare grinding and polishing slurries

1. Grinding and polishing slurries should be made and stored in wash bottles. Label each wash bottle to indicate which slurry it contains.
2. *To prepare the grinding slurries*: Select which grit size you want to use: 30, 15, or 5 micron. Scoop enough grit powder to fill the wash bottle about ⅓–¼ full and then fill the rest of the bottle with water. Shake well before each use, ensuring the grit and water are combined.
3. *To prepare the polish slurry*: The micro-polish liquid can be combined with water in a 1:3 polish:water ratio (instructions are usually provided on the bottle). When using the polish slurry, notice that a little goes a long way and that spraying excessive amounts of slurry onto your pad doesn't help speed up polishing, but only wastes the slurry.

B. Grinding the first (non-sulcus) side

Important tips to remember when grinding

- Grind at a relatively low wheel speed.
- Frequently check the progress of your grinding by looking at the slide under a light microscope. It can be tricky to determine whether you are looking at the otolith surface or at the CB covering the otolith; nevertheless, it's important to take time to ensure that you're seeing the sample correctly. One approach to help you identify which surface material you're seeing on your sample (CB or otolith) is to look at the area outside your otolith. The area surrounding the otolith will be CB. Look at the texture of the surface in the CB area. Compare the texture of the CB surface to the texture of the otolith surface. If the textures differ, you most likely have removed the CB above the otolith and are viewing the otolith surface.
- While grinding, make sure the grinding pad is always wet and that you use plenty of slurry so as to cushion the otolith and reduce friction between the otolith and the grinding pad. If the pad becomes too dry it can cause the otolith to snag, chip, or break. Indicators that the grinding pad is too dry include:
    - The slide begins to stick to the grinding pad (though this can also happen if the grinding pad is too wet).
    - You are unable to smoothly move the slide across the pad surface as it is grinding.

  If this happens, add more slurry and perhaps a little water. With practice, you'll discover the ideal amount of water and slurry that works best for you.
- If the grinding pad tears while you're working, it's best that you replace it. Grinding on top of a tear can break your otolith or cause it to be ripped from the slide.
- While grinding, it's important to observe the entire otolith surface; however, you won't actually use the entire surface for image analysis. During the image analysis process (marking and measuring otolith increments), only increments within the "area of interest" will be marked (Figure 10). We mark and measure increments within this area because it tends to contain the highest-quality increments. Therefore, as you're grinding, take time to notice how the entire otolith surface is grinding, but more specifically focus on producing high-quality increments (increments that are clear and easily visible) within the area of interest.

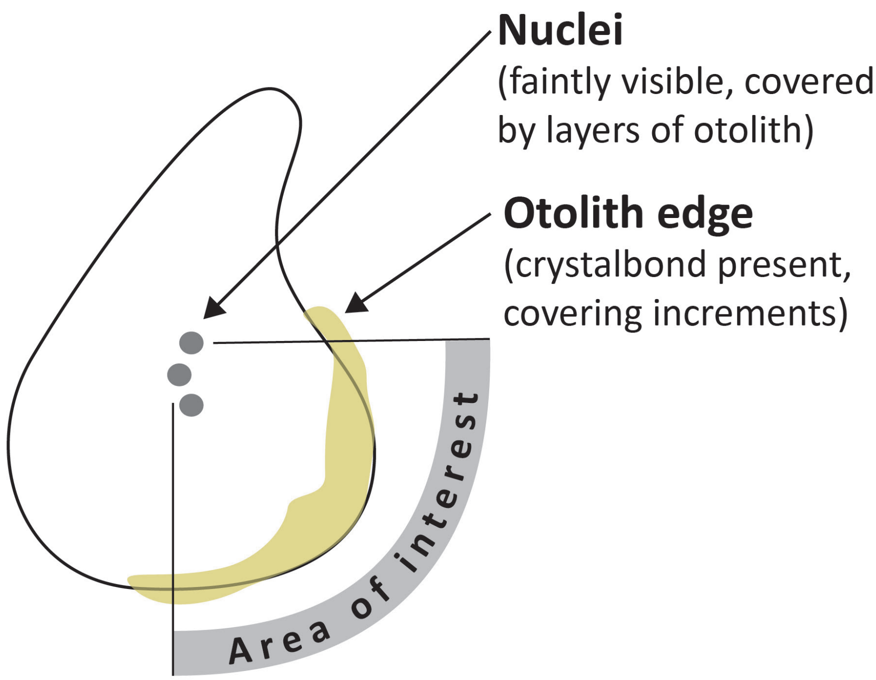

Figure 10. Otolith sample and indicators depicting when to stop grinding.

Steps

1. The objective for grinding the first side of the otolith (the non-sulcus side) is to remove enough otolith material so that the otolith becomes transparent, allowing you to clearly see the nuclei and increments after you flip and grind the second side. It is important that you do not grind too much on the first side; doing so will cause the otolith to become too thin, possibly grinding through all the increments or making it difficult to detect or read the increments. Step 6 will define at what point you should stop grinding on the first side.
2. Place your slide in the grinding jig. (Note that using the jig is optional. Sometimes freehand grinding is preferable. The jig is particularly helpful when polishing large otoliths of adult fish. You will have to try grinding freehand as well as in the jig to decide which works best for you.)
3. If your otolith is embedded within resin, continue to Step 4. If it is just CBed to the slide, skip to Step 6.
4. Begin grinding your resin blocks with 30 grit and a TexMet Buehler grinding pad.
5. Grind using the 30-grit slurry until the otolith surface is slightly exposed. Stop grinding once you have ground through the resin. Even if it appears that you've only ground through a tiny section of resin, stop with the 30.

Note: We grind with 30 because the grit is large and therefore quickly grinds through resin. Make sure to stop grinding once the otolith surface is slightly exposed (resin has been ground away), because grinding too much with 30 may gouge, crack, or remove too much material from the otolith.

Pay special attention to notice where initial grinding contact occurs on the otolith surface (i.e., where on the otolith the resin is first removed). Contact should be centrally located over the core area: if grinding occurs on the outer right edge, then the otolith is raised too high on the slide; if grinding occurs just left of the core, then the otolith is set too low on the slide. It's very helpful to stop and reposition your otolith if the initial grinding doesn't occur correctly. To reposition the otolith, place the slide on the hot plate. Once the CB begins to soften, use a probe/small syringe to gently press down on the side of the otolith that appeared to be grinding away first (in an effort to make the otolith surface as flat as possible and therefore allow you to grind the otolith on a flat plane).

6. Next, grind the otolith using 15-grit slurry and a TexMet Buehler grinding pad. As you grind, remember to keep the grinding pad wet at all times and periodically spray more slurry to keep the pad surface smooth. Grind with 15 grit until you begin to see the otolith nuclei. Make sure you stop grinding while resin or CB is still covering the edge of the otolith (the nuclei should have many layers of otolith material over them, so they will appear as tiny, black blobs—if you focus in further, you may see defined increments around the nuclei; see Figure 10). While grinding, it's important to look at the entire otolith surface and observe how the whole surface is grinding (i.e., does the entire otolith surface appear to be grinding evenly or are some areas of the otolith being ground faster than others?).
7. Further grind your otolith sample using 5 grit and a TexMet Buehler grinding pad. Before discussing how to grind with the 5-grit slurry, here are a few thoughts about this slurry:
    - You can continue using the same pad as in Step 6; however, make sure to thoroughly rinse the pad before you start grinding with 5 slurry.
    - Grinding with the 5 slurry is nice because it slowly grinds through otolith layers, enabling you to grind with some finesse and lessen the chances of overgrinding and destroying samples.
    - This is the last slurry that you'll grind with before flipping the otolith. (Before flipping the otolith, some labs grind with a 3.0 slurry because it grinds even more slowly than 5 and can remove very small layers.)

How to grind with the 5 slurry

The objective when grinding with 5 is a balancing act: you want to remove enough otolith layers so that increments in the core and edge are clear, but it's important to not remove too many layers, especially over the edge increments (Figure 11).

The balancing act

- Be certain to grind away enough of the otolith so that the nuclei and edge increments are visible.
- Don't overgrind the otoliths. Nuclei and edge increments can be ruined from grinding too much, rendering the samples unusable. (Generally it's preferable not to grind away any nuclei. However, in some situations, it may be necessary to grind away some outer nuclei in order to increase the overall clarity of the increments. In such cases, it's all right to lose the outer nuclei because they aren't necessary for image analysis.)

While grinding with the 5 slurry, monitor the grinding progress at both the core and edge regions. Stop grinding with 5 while both the nuclei and the edge are still covered with some otolith layers and CB (respectively). At this point in the process, the edge is thin, and if all the CB were removed you would run the risk of grinding away the outer increments.

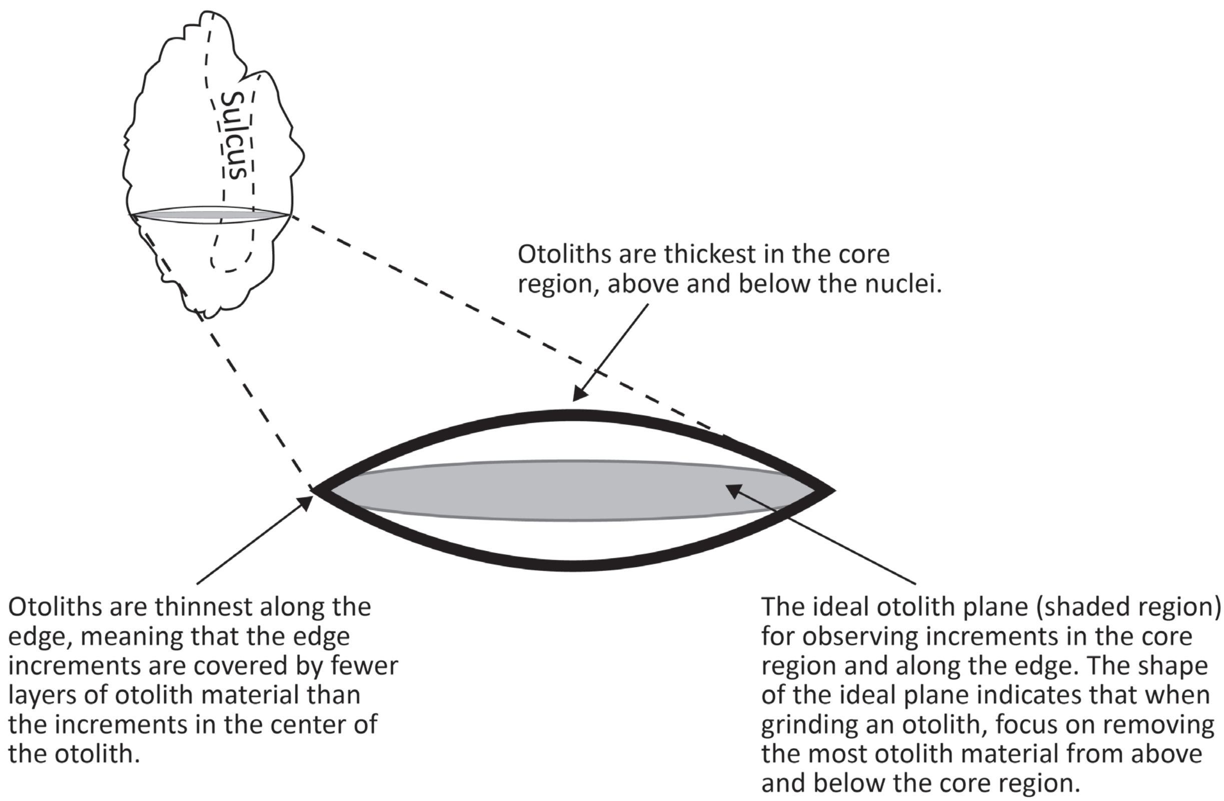

Figure 11. Otolith cross-section, illustrating the elliptical shape of the cross section; and ideas to consider when processing an otolith.

8. Polish the otolith with the micro-polishing pad (usually it's white and soft). Wet the pad with water and 1.0 alumina polish and grind for about 15 seconds. The polish will fill in any tiny gaps on the otolith surface and improve the clarity of the increments.

C. Flipping the otolith

1. Turn on the hot plate to a low setting and place the slide onto the hot plate. Once the CB begins to melt, remove the slide from the hot plate. Quickly—but gently—use forceps or the tip of a small syringe to flip the otolith (or the resin block with the embedded otolith).
2. Once flipped, use a probe to gently push down on the resin block (or, if no resin block, the otolith itself) to ensure it lays flat against the slide surface. (It may be helpful to use a microscope to position the otolith after it's flipped.)

D. Grinding the second (sulcus) side

1. Grind the otolith with the 15 slurry and a TexMet Buehler grinding pad. Stop grinding when the nuclei first become visible and while CB still covers the otolith edge. After flipping, the nuclei may be visible immediately—in which case, grind a little with the 15, just enough to remove the CB over the otolith core region. Grinding on the second side can go quickly, so check your progress often.
2. Once the otolith core region is exposed, grind further with the 5 slurry and the same grinding pad as in Step 3 (thoroughly rinse the pad before using the 5 slurry). Grind until the nuclei are clearly visible and the edge increments are clear and distinguishable within the area of interest.
3. Polish your sample with 1.0 alumina micropolish and the MicroCloth pad. Polish for about 15–20 seconds.

Otolith Image Analysis Protocol

This protocol outlines steps for conducting image analysis on juvenile salmonid sagittal otoliths.

Capturing the otolith image (taking a picture of an otolith)

The objective when capturing an otolith image is to get a picture that is in focus, has good clarity, and contains both the otolith nuclei and an edge. (If an otolith sample is so large that its nuclei and edge cannot be captured within a single image, it may be helpful to tile images together.) It's helpful to position your computer next to the imaging scope and camera (Figure 5).

1. Turn on the computer and open Image Pro.
2. Place the otolith sample under the light microscope. Bring the otolith into focus using either the 10× or 20× objective. It's most important that the otolith region within which you will be measuring increments—the area of interest—is in focus, and that increments near the edge are easily identifiable. To determine the area of interest for each otolith sample, you'll need to know if the sample is a left or right otolith and which side is the sulcus side; see Figure 12.
3. In Image Pro, open the **Video/Digital Capture** window: Click **Acquire**, then **Video/Digital Capture**, and then **Preview** to see a live view of the otolith on your computer monitor.
4. Looking at the live preview, select an objective on the microscope such that in the viewing window you can see the otolith area of interest, containing both the otolith nuclei and edge. Try to use the highest objective possible. It may be helpful to rotate the camera so as to fit the area of interest within the view. Typically I use the 20× objective, but otolith size will dictate which objective you use.
5. Save the otolith image once you've obtained a good picture of the sample. To do so, click **File** and **Save As**. When creating a file name for an otolith sample, it's helpful for the name to contain both the fish sample ID and the objective used to take the picture. Saving the objective within the image file name is particularly helpful in the following steps when you calibrate your image, because you can quickly (and assuredly) recall which objective was used to capture the image.

Analyzing the otolith sample (marking/measuring otolith increments)

Objective: To draw a radius across the otolith (from the nuclei to the otolith edge) and to mark increments along this line in a way that accurately represents increment widths throughout the entire otolith. Identifying increments can be difficult (depending on the quality of the otolith), and it takes practice getting accustomed to seeing increments and distinguishing between real and false increments.

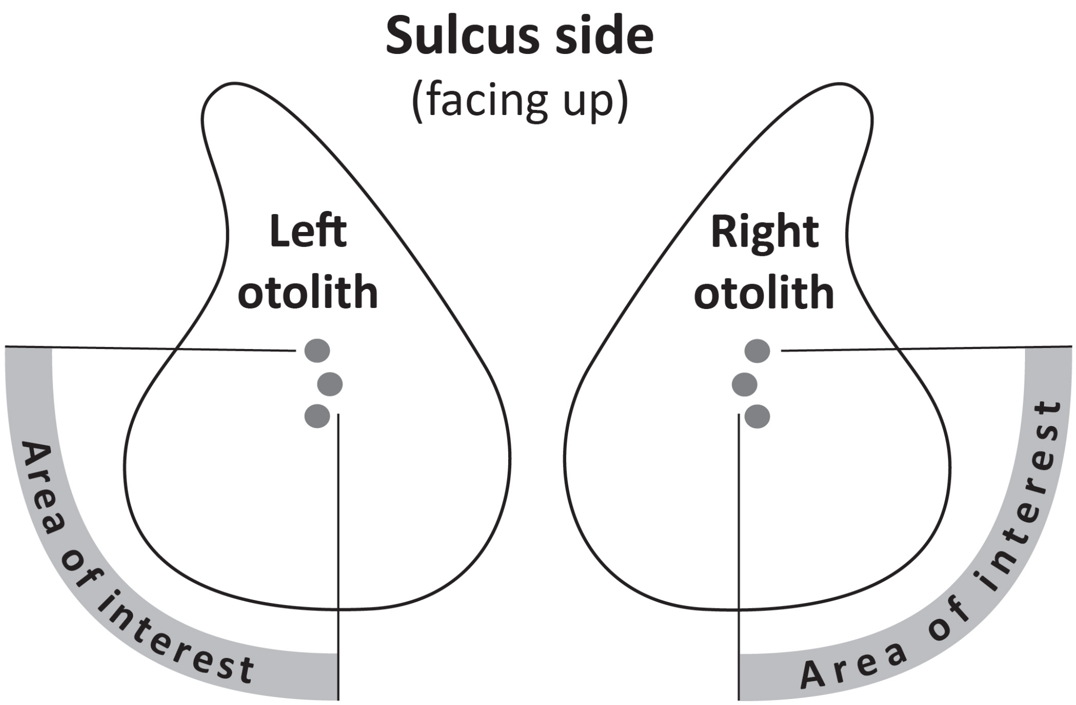

Figure 12. Sulcus side views of left and right otolith samples, identifying the areas of interest.

The following information about marking otolith increments is organized in two parts. Part A outlines how to mark increments on an otolith image using Image Pro software, including how to draw a line to measure the otolith radius and how to create and use markers for measuring otolith increments. Part B presents helpful ideas to consider while analyzing an otolith image, such as tips for how and where to draw the otolith radius and how to distinguish between real and false increments.

A. How to mark an otolith sample

1. Open the otolith image you want to measure. Select a preferred nucleus to begin drawing the otolith radius from. The otolith radius line serves two purposes: 1) it provides a length measurement for the otolith radius, and 2) it's the line along which you will mark/measure otolith increments.
2. To create the otolith radius, open the **Caliper** window (**Measure**, then **Caliper**). Along the left side of the **Caliper** window, in the top row of boxes, click the second box in from the left (the box with a diagonal black line). Use the mouse to position the crosshairs over the preferred nucleus you've selected to use. Next, draw the radius by pressing and *holding* the left-click button. Continue to hold the left-click button as you drag the crosshairs across the otolith (from the nucleus to the edge). Make sure to extend the radius past the otolith edge. Once the radius is past the otolith edge, release the left-click button to stop drawing.

3. To mark the otolith increments, you'll first need to create "edge detectors." Edge detectors are small tic marks that are used to measure otolith increments. To create an edge detector, click **New** within the **Caliper** window (on the left side of the window, beneath a white box labeled **Edge Detectors**). A new window titled **Edge Detector** will appear. Under **Type**, make sure that **Derivative** is toggled, then click **Select.** You should now see more of the **Edge Detector** window become interactive. Within this window, you only need to change the "sensitivity threshold" to **100** in the box next to **Sensitivity threshold**.
   FYI: The default settings within **Edge Detector** are: **name** = **peak**, **label** = **A**, **color** = **red**, **detect position of** = **peak**, and the red line on the graph is centered between **offset** and **pixels**. Although it's not necessary to change these settings, you can change **name**, **label**, and **color** to your liking. Click **OK** when you've finished making an edge detector.
4. Within the white **Edge Detector** box you should see an edge detector name that is highlighted in gray [e.g., **Peak(A)**]. To begin marking increments, click the box with a red dash within the **Caliper** window. Move your mouse arrow over the caliper line and left-click (on the caliper line) to mark the increments. You can remove any edge detector mark by left-clicking on it. Sometimes, while trying to mark increments, you may notice that as you click to add a new mark, an adjacent mark is erased. This seems to occur if the increments are very close to each other. It may be helpful to zoom in and out of the otolith image in such cases.
5. Once a few increments are marked, save your work and then continue marking the remaining portion of the otolith. Periodically save your work—it's terribly frustrating to lose work in the rare case that your computer freezes.
   To save the radius line and increment markers: Within the **Caliper** window, click the **Input/Output** tab, click **Save**, enter the appropriate location and file name, and click **Save**.

Note: Before you begin marking increments or save the caliper line, be certain that its placement is correct. Once you begin drawing edge detectors, you cannot change the caliper line; trying to do so will delete the edge detectors.

B. Key points to consider when analyzing an otolith sample

1. *Choosing which nucleus to use.* Choose a nucleus from which you can draw a radius line that doesn't pass through areas of the otolith where increments are inaccurate or unidentifiable (see points 4 and 5, below). In an effort to do this, I often use a nucleus that is in the middle of the core (the top of the core is the area nearest the rostrum; the bottom of the core is nearest to the post-rostrum). Sometimes I'll use a lower nucleus, one that is in the lower region of the core, if the increments outside the middle nuclei are of poor quality or difficult to mark. Typically, I try not to use the uppermost nuclei at the top of the core, because the surrounding increments are often of poor quality and not representative of other increments around the otolith. Additionally, upper nuclei lying outside the main region are secondary nuclei and should be avoided, as they will produce incorrect radius measurements.
2. *Drawing an accurate radius line.* Take time when deciding where to place the radius line. Be certain that the location you choose accurately represents the otolith radius. This can be tricky to do because the otolith edge is curvy; thus, it's important to identify and draw the radius through an area where the otolith edge appears to be an average distance from the nuclei (Figures 13 and 14).

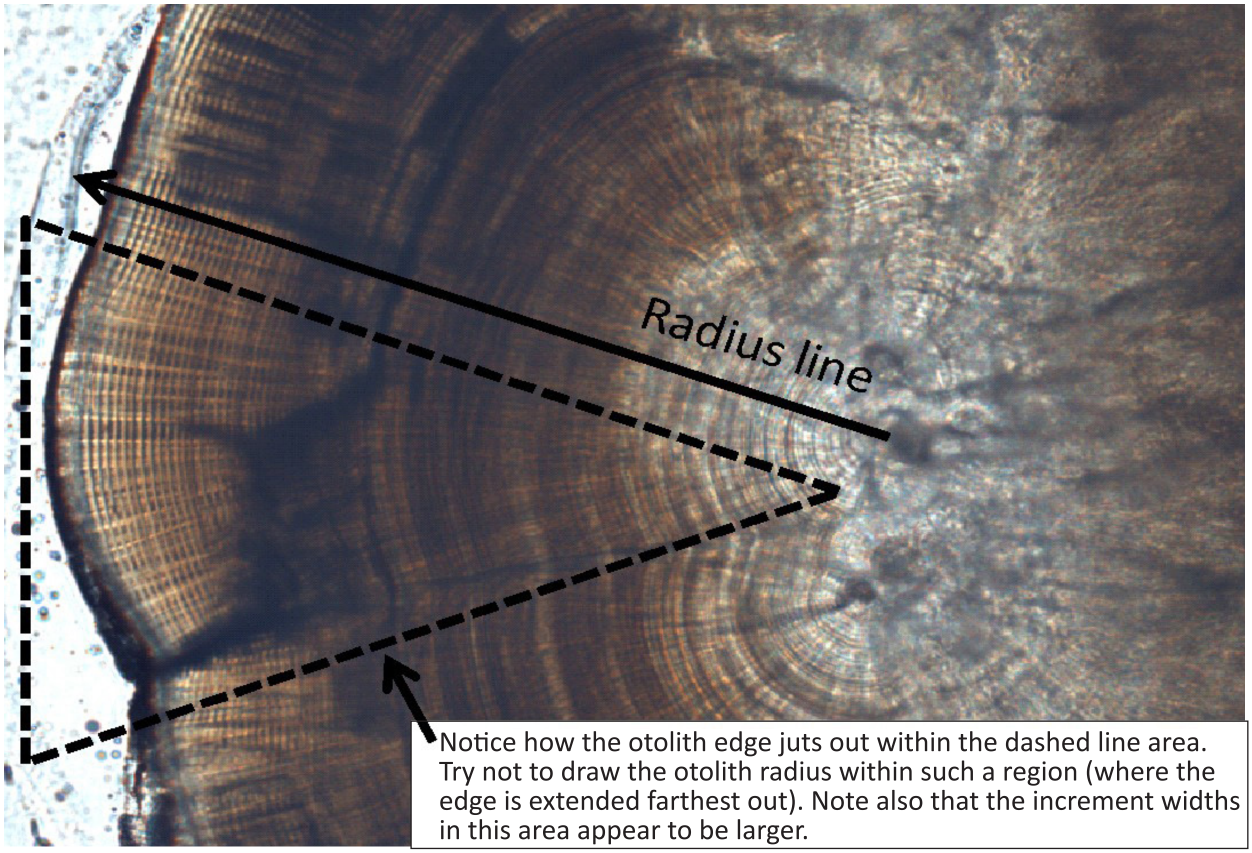

Figure 13. Otolith showing the radius line and illustrating a problematic area (enclosed within the dashed line) to avoid when drawing the radius.

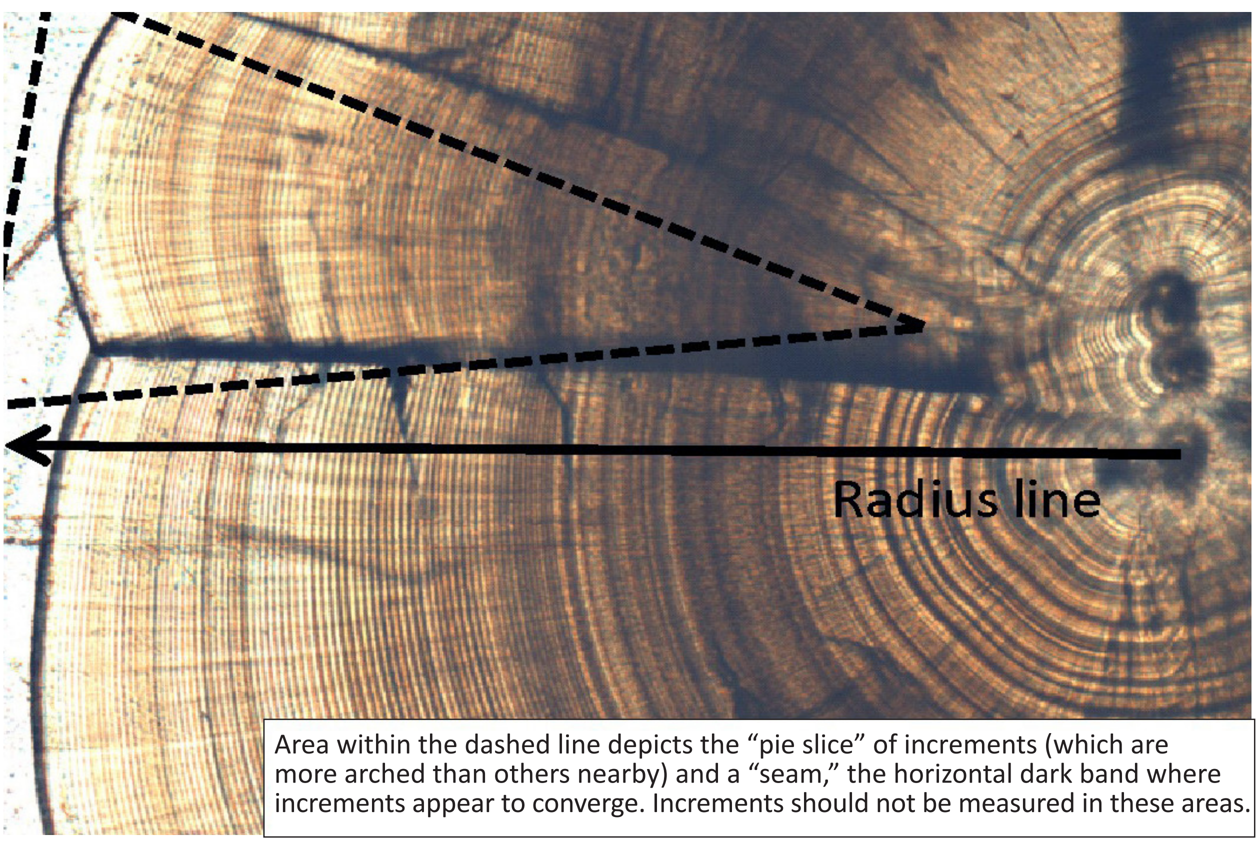

Figure 14. Otolith showing the radius line and a troublesome area (enclosed within the dashed line) to avoid during analysis.

3. *Drawing the radius line perpendicular to otolith increments.* Try to draw the otolith radius perpendicular to the increments in order to get the most consistent and accurate increment-width measurements. This is not always possible; however, try your best.
4. *Ensuring accuracy and precision when marking otolith increments.* As you extend the radius line from the nucleus to the otolith edge, be careful that the increment widths (the distances between adjacent increments) in the region the radius passes through are similar to increment widths around the whole otolith. Be careful to avoid marking increments when they resemble any of the following:
   i. With just a quick glance at most otolith samples, you can easily see that increment widths are not consistent around the entire otolith. For example, if you follow two adjacent increments around the otolith you may see areas where their increment width becomes compressed or widens. If you mark increments in either of these two areas, the increment width measurements you obtain will inaccurately describe otolith growth (by extension, they will also inaccurately describe fish growth at that stage in the fish's life). Therefore, to reduce bias that may result from how we count and measure increments, we do our best to sample each otolith in the same section.
   ii. Increments that appear to grow in different directions. Sometimes on an otolith you may see something that looks like a seam, positioned perpendicular to the increments. In such areas the increments seem to be growing in different directions and appear to converge, causing increment widths to vary and, at times, reducing the clarity of increments.
   iii. Look for regions of the otolith (often shaped like a slice of pie) that begin at the upper or middle nuclei and radiate out to the otolith edge. These areas often contain increments that appear more arched than the surrounding increments. These overly arched increments seem to form a growth pattern that's different than other increments, so marking them may lead to inaccurate otolith measurements. (What to look for: a pie piece [containing increments that look more arched] that appears to have been jammed into the otolith.)
5. *Distinguishing between real increments and fake increments.* Determining if increments are real or fake can be one of the most challenging aspects when marking otoliths. When otoliths are ground well and have good clarity, it's much easier to distinguish real increments from fake increments. Often, regardless of how well polished the otolith is, some increments will require more scrutiny to determine if they are real or not. Here are a few strategies I use to help decide which increments are real:
   i. Find a good otolith sample, one that contains increments that are very clear and easy to identify. As you analyze the rest of your samples, use the good otolith as a reference for comparison when you come across questionable increments.
   ii. Sometimes, after polishing an otolith, its increments are not at the surface of the otolith, but instead they may be covered by a few layers of otolith material. Such otolith material may reduce the clarity of your increments, to the point of blurring them and causing two increments to appear as one; alternatively, one increment may appear separated into two increments. In such troublesome areas, look at the questionable increments through the microscope and slowly move the focus in and out. As the focus changes, so too will the contrast between the increments. Typically with real increments, as you change the focus you will

see a strong contrast of black and white between the real increments. The real increment will appear black in contrast to the white appearance of the otolith material between each increment. If the increments are fake, as you focus in and out you may see slight differences in the contrast between what seems to be separate increments, but you won't see a sharp black-and-white contrast that seems to jump out at you, as you would if the increments were real.

iii. If you see very small, tiny increments, it may be a sign that increments are not what they seem. This idea may be confusing, since otolith increments are naturally very small; however, at times you may see patches of increments that are exceptionally small. When you see such increments, compare the small increments (in question) to increments on your reference otolith (from Step i). If the smallest increments on your reference otolith are all larger than the tiny increments that you're working with, most likely the tiny increments are not real. In which case, try following the tiny increments around the whole otolith to see if they're larger anywhere else on the otolith. You may also try focusing in and out, as in Step ii.

Creating calibrations for each microscope objective

Objective: Create calibration files for each objective (on your microscope) using a ruled stage micrometer. A ruled stage micrometer is a slide with a ruler printed on the surface. The micrometer we use shows a 2-mm scale, marked in 0.01-mm and 0.1-mm divisions. 0.1 mm = 100 microns (µm), 0.01 mm = 10 µm.

1. Open Image Pro. Place the micrometer slide on the microscope stage. Select an objective, one in which you'll need a calibration—we only use 10×, 20×, and 40× to take pictures of our otoliths. Bring the ruler on the micrometer into focus.
2. Within Image Pro, open the image preview (**Acquire** > **Video/Digital Capture**). Preview the micrometer (on the computer monitor) and bring the ruler into focus.
3. Within Image Pro, click **Measure**, **Calibration**, and **Spatial Calibration Wizard**. In the **Create spatial calibration** pop-up window, ensure that **Create reference calibrations** is toggled. Click **Next.**
4. Within the same window, a new screen will appear; again, click **Next.**
5. The new screen will ask if you want to "test capture." We just did that in Step 2, so you don't need to test it again. Click **Next.** However, you may have to click **Test Capture** before you can click **Next**; if this is the case, click **Test Capture**.
6. Nothing needs to be entered into the "use magnifier" field; simply click **Next.**
7. Type the objective name inside the "Objective to calibrate" field. (Generally we don't use the "add/edit objectives" section, so I'm not sure what role it plays in calibration.) Click **Next.**
8. Read and follow the instructions on the screen. (Basically, all you need to do is click **Capture**, since you've already focused the micrometer.)
9. Read and follow the instructions for drawing reference lines. Draw several lines (about 8 or so) of various lengths (both very small and long lines). Drawing several lines helps to improve the accuracy and precision of your calibration. After drawing the reference lines, click **Next.**

10. Select either **Create Another** (to create calibrations for the other objectives) or click **finish** to stop making calibrations.
11. Test the calibrations to make sure they are correct. To do so, take images of the micrometer using different objectives. For each image, open and apply the correct calibration. (Important: In the **calibration** window, within the "Unit" field, check that it reads "µm" or "microns.") Now draw lines on the micrometer image to determine if the calibration is accurate; click **Measure**, then **Measurements**. Click the middle button in the second row (it should have a picture of a black diagonal line). Draw a few lines. If the measured lengths are not correct, redo the calibration process.

Exporting otolith data

1. After you've finished analyzing your otoliths, the next step is to export the otolith measurements to Excel (or another program of your choosing). I've found it to be most efficient to export the otolith data after I have analyzed all the otoliths in a sample group, instead of exporting data after analyzing a single otolith.
2. The first step in exporting otolith data is to ensure that the increment measurements are correct. To do so, it's important that you apply the appropriate calibration to each otolith image before you "load" the increment markers (a.k.a. the edge detectors) onto the otolith image. (We can't expect to get correct data if we're using an incorrect calibration. So, before opening the increment marker file and loading the increment marks onto the otolith image, we need to tell Image Pro which objective was used when we took the otolith picture. We use the **Calibration** function to do this.)
3. Here I'll explain what I've found works well for me, but naturally you'll develop your own approach over time. Once all my otoliths are analyzed and all the data are saved (including all the otolith images, caliper lines, and edge detectors), I close down Image Pro and reopen it. (It's like starting with a blank slate.)
4. Open the otolith image for which you want to export the data. Click **File** then **Open**, find your image within the pop-up window, and click **open**.
5. Next, apply the appropriate calibration to the otolith image. Click **Measure**, **Calibration**, and then **Spatial…**. A **Create Spatial Calibration** pop-up window should appear. In the top of the window, to the right of **Name**, click the upside-down triangle to open a drop-down file from which you will select a calibration to use for this image (select the calibration that matches the objective used to take the otolith image; this is when it is helpful to have included the objective as part of the image file name). Click **Apply** and then **System**.
6. Now you will load the increment markers onto the otolith image. Click **Measure** and **Caliper**. Within the **Caliper** window, click the **Input/Output** tab and click **Load.** Open the caliper file that matches the otolith sample. The caliper line and increment markers should appear on the image.

7. To tell Image Pro which measurements you'd like to export, return to the **Caliper** window and click the **Measurements** tab. Within the tab, click the **Measurements** button. At the top of the **Measurements** pop-up window, you should see the edge detectors that you used to mark increments. Below, there should be five measurement options to choose from. The three most-useful options and what they measure are:
   i. *Distance from sampler's origin*: Measures the distance from the origin to each increment marker (the origin is the point at which the caliper line starts). Because the caliper line begins at a nucleus, this function measures the distance from the nucleus to each increment marker. For example, if you choose this measurement option and you've marked 50 increments, then you'll have 50 measurement values, one for each increment marker.
   ii. *Distances between markers of an edge detector*: Measures the distances between increment markers, which means it's measuring the distance between each increment (a.k.a. the increment widths).
   iii. *Distances between markers of two edge detectors*: Measures the distance between two different edge detectors (a.k.a. increment markers), similar to the previous option; however, you can only use this if you have more than one edge detector.

   To select multiple measurement options, click **Add**, then toggle a measurement option. Repeat these steps to select more measurement options. When you've added enough measurement options, click **ok** to exit this window.

Note: Generally I select two measurement options for each otolith, both i) and ii).

8. Click the **Input/Output** tab, then **Options**. If you want to export data to Excel, make sure it is listed in the **Target Program** line. Adjust **Sheet**, **Row**, **Column**, and the toggles as you'd like (indicating how additional data will be added to the Excel worksheet). Click **OK** to exit.
9. Step outside of Image Pro for a moment and open Excel. Open a file or worksheet to receive the exported data. Keeping the file/worksheet open, return to Image Pro.
10. Within the **Caliper** window, on the **Input/Output** tab, press **Send Data**.
11. Return to Excel. In the row above the recently imported otolith data, type in a data label (include such information as the fish sample ID and the collection date).
12. Repeat these steps with the remaining samples. Remember to keep Excel open so that all the data (from an entire sample set) will be imported into the same worksheet.
13. Once all the data for a sample set have been imported into Excel, it's helpful to organize them so that they are easy to read, understand, and use. Table 1 is an example of an otolith data spreadsheet. This spreadsheet may not work for all datasets, but it may provide some ideas of how to get started on organizing your own data. After I've finished importing all the data for a sample set, I find it helpful to create a summary of that data and store it within the same worksheet.

Note: Otolith radius is the last measurement in the "Distance from sampler's origin" data column. See Table 1.

Table 1. Example of an otolith data spreadsheet. Summarized data are in the upper-left corner. In order to save space, a few rows of data have been removed.

Sample ID	Collection date	Otolith radius	Fish FL[a]			
Fish1	09/01/15	566.49	78			
Fish2	09/01/15	598.44	85			
Fish3	09/01/15	518.32	79		Fish1	
Fish4	09/01/15	515.43	72		A	A – A
				1	427.40	3.65
				2	431.05	3.65
				3	434.70	3.65
				...	...	...
				43	558.93	2.86
				44	561.80	4.69
				45	566.49	

Distances between edge detectors (increment widths)

Otolith radius is the last increment in the data column

Distance from sampler's origin

[a] Fork length.

Quality assurance of otolith increment data

Precision

To assess the quality of otolith increment data, we repeat the otolith marking procedure (i.e., Analyzing the otolith sample) for a random subset (approximately 10%) of the otoliths. For this subset of otoliths, we use the original digital image of the otolith but we do not reference the previous otolith marks and measurements. Preferably, the person who marked and measured the otoliths the first time will not repeat the otolith marking procedure. However, if a different person is not available, we recommend that the replicate otolith marking and measurements be conducted on a different day, to ensure no bias from memory.

Once the subset of otoliths has been measured twice, we determine the average increment width across the last seven increments for both measurements of every otolith. We then compare the averages between replicate measurements using Student's t-test. The Student's t-test allows the independent readings of the same otoliths to be compared to confirm whether both provide similar results. If no significant difference is observed between replicate measurements, then we conclude that our otolith measurements have a high repeatability and are thus of good quality. However, if significant differences are detected between replicate measurements, then we follow a three-step process to improve otolith measurement quality:

1. First, we identify which otolith(s) in the subset show the greatest replicate measurement variability by calculating differences between replicate measurements.
2. Second, for the purpose of identifying where the deviations in increment marks between replicate measurements arose and how to revise the otolith measurement(s), we compare otolith increment widths and increment markings on the otolith digital images for those otoliths that have the greatest differences between replicate measurements.
3. Third, we repeat the Student's t-test on the revised measurements from the subset of otoliths. If this test fails, then we repeat Steps 1–3 on the same subset of otoliths again. If the test passes, then we repeat the above test on a new subset of otoliths.

Bias

Bias demonstrates the degree to which the measured value represents the true value. Each otolith will be read without any knowledge of which site the fish was sampled from. Bias or accuracy of samples will be minimized through consistency in the measurement protocols, ensuring the increment being measured is in optimum focus, and ensuring the otolith is mounted so that the incremental plane is as close to horizontal as possible.

Completeness

Completeness is the ratio of usable data from the otolith analyses. It is fully expected that all otoliths will be processed and read, producing a reliable data point from each fish.

Representativeness

The sampling design is aimed at representing average daily growth across a range of days. The otolith samples will be properly stored and processed within an appropriate timeline.

Comparability

Comparability is the similarity among different datasets for use in combining or comparing data. The methods used in this analysis follow similar protocols as previous studies.

References

Secor, D. H., J. M. Dean, and E. H. Laban. 1991. Manual for Otolith Removal and Preparation for Microstructural Examination. The Electric Power Research Institute and the Belle W. Baruch Institute for Marine Biology and Coastal Research:84.

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